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Effects of Selected Herbicides on the Germination and Infection Process of *Puccinia lagenophora,* a Biocontrol Pathogen of *Senecio vulgaris*

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The herbicides 2,4-D, glyphosate, linuron, and MCPP at rates of 1X (recommended field rate), 0.25X, 0.025X, and 0.0125X were evaluated *in vitro* for their effects on the rust fungus *Puccinia lagenophorae,* a biocontrol agent for the annual weed *Senecio vulgaris.* Herbicides applied at 1X and 0.25X completely prevented aeciospore germination. Glyphosate was toxic even at 0.0125X and 0.025X. Aeciospores germinated in linuron, 2,4-D, and MCPP at 0.025X and 0.0125X at rates similar to the water control. Abnormal germ-tube growth was observed with 2,4-D at 0.25X and 0.025X, with linuron at 0.025X, and with glyphosate at 0.0125X. Further *in planta* studies were perfomed with two inbred lines of *S. vulgaris* inoculated with aeciospores of *P. lagenophorae* and treated with water, linuron, and 2,4-D at 0.025X at different times of application. Quantitative analysis of the infection process revealed that both herbicides reduced spore deposition on the leaves and altered leaf morphology. The herbicides had no effect on disease severity at this low rate although linuron significantly reduced the formation of infection peg. Timing of herbicide application had no influence on the infection process, and the effect of the herbicides on fungal development did not differ between the two plant lines. Thus, the herbicides applied at 0.025X did not increase plant susceptibility to the rust fungus, and the rates of 1X, 0.25X, and 0.025X would prevent, inhibit, or delay fungal development. Therefore, joint application of *P. lagenophorae* with these herbicides to control *S. vulgaris* cannot be recommended.

***Key Words: Senecio vulgaris;* common groundsel; *Puccinia lagenophorae;* biological control; component analysis; herbicides; linuron; 2,4-D; compatibility.**

INTRODUCTION

Often a pest species is host to a number of natural enemies and this natural association of interactive agents can be exploited to achieve integrated pest control. Rather than as a replacement for chemical pesticides, biological control of weeds is gaining increasing importance as a viable part of well-designed, integrated pest management systems (Jacobsen and Backman, 1993; Müller-Schärer *et al.,* 2000). Fungi figure prominently among potential biocontrol agents of major agricultural pests. Integration of selected biocontrol measures into general integrated pest management strategies requires that biocontrol agents are compatible with pesticides used in the target crop. Examples of interactions between biocontrol agents and pesticides have been dealt with in various review articles (Altman *et al.,* 1990; Charudattan, 1993; Christy *et al.,* 1993; Papavizas and Lewis, 1988; Smith, 1982).

Chemical pesticides may have a major impact on the effectiveness of biocontrol agents. Since herbicides are used quite extensively in agriculture, the potential for their interference with pathogen-weed interactions is quite apparent. They may suppress the biocontrol agent in the field by limiting its establishment below a desired population density and slowing the build-up of an epidemic in the field. Therefore, in order to successfully implement biocontrol, growers must know whether a biocontrol agent is compatible with the various pesticides used in the crop.

Some herbicides and growth regulators with specific chemistries and molecular modes of action have been shown to act synergistically with biocontrol agents to enhance weed control (Christy *et al.,* 1993). Sharon *et al.* (1992) demonstrated that the suppression of biosynthesis of a phytoalexin in *Senna* (=*Cassia*) *obtusifolia* (L.) H. S. Irwin & Barneby by the herbicide glyphosate at sublethal rates led to subsequent increase in susceptibility of this weed to the mycoherbicide *Alternaria cassiae* Jurair & Khan. Another example of synergy

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between biopesticides and chemicals is in the application of *Puccinia canaliculata* (Schw.) Lagerh. in combination with the herbicide paraquat to achieve 99% control of *Cyperus esculentus* L., compared to only 60% control with the rust alone (Phatak, 1984). Timing of herbicide application might also be a major factor in integrating biocontrol pathogens and other chemicals in attempts fo find synergy. Tank-mixture of a biocontrol agent and agricultural chemicals can be detrimental to the living organism but a sequential application may prevent inhibition of the pathogen's growth or activity. In the case of *Colletotrichum gloeosporioides* (Penz.) Sacc. f. sp. *aeschynomene,* marketed as the microbial herbicide Collego for the control of *Aeschynomene virginica* L., the tank-mixture of the fungus with the herbicides propanil and 2,4,5-T, and the fungicide fentin hydroxide inhibited fungal colonization. Although there was no synergy in this system, infection and disease development were successful when the pathogen was applied before the chemical herbicides and the fungicide (Smith, 1986).

The weed pathosystem *Senecio vulgaris* L.-*Puccinia lagenophorae* Cooke was chosen as a research model to test a system-management approach of biological weed control (Müller-Schärer and Frantzen, 1996; Müller-Schärer and Scheepens, 1997). This approach aims at the reduction of competitiveness of the annual weed *S. vulgaris* (common groundsel) by stimulating disease epidemics with a relatively small initial inoculum source (Frantzen and Hatcher, 1997; Frantzen and Müller-Schärer, 1998). A survey carried out in horticultural crops, in nurseries and ruderal sites in Switzerland indicated that despite abundant use of pesticides, especially fungicides, *P. lagenophorae* still occurred at 60% of the sites investigated (Wyss, 1997). These fungicides were applied over the whole growing season and rust incidence was observed predominantly in early autumn. The rust fungus either is not controlled by fungicides or survives locally and the disease becomes epidemic later in the year.

Wyss and Müller-Schärer (1999) have demonstrated the existence of race-nonspecific quantitative resistance in *S. vulgaris* to *P. lagenophorae.* This resistance is manifested during the most sensitive transition in the infection process, namely at the stage of penetration-peg formation when the fungal development was most reduced.

The present study had two objectives. The first was to determine the effect of the herbicides linuron, 2,4-D, glyphosate, and MCPP at their recommended field concentrations (1X), and at 0.25X, 0.025X, and 0.0125X, on the germination of aeciospores of *P. lagenophorae* (*in vitro* study). These herbicides were selected because they (i) are commonly used to control *S. vulgaris* in horticultural crops in Europe, (ii) represent different modes of action, including residual, contact, nonselective broad-spectrum, and growth-regulating effects, respectively, and (iii) have different degrees of effectiveness on *S. vulgaris* (Baumann, 1994). The second objective was to determine the effects of linuron or 2,4-D at a sublethal rate of 0.025X on the infection process by the rust fungus in two inbred lines of *S. vulgaris* (*in planta* study). The herbicides were applied either 48 or 0 h before inoculation with the rust fungus to represent sequential and tank-mixed applications of the herbicides and the pathogen. Component analysis was used to quantify the effect of the herbicides on the infection process and to determine a potential increase in host-plant susceptibility from the herbicide application.

MATERIALS AND METHODS

Plant and Fungal Material

Two inbred lines of *S. vulgaris* grown for five generations were used. The lines were from Unterehrendingen, Switzerland (pCH I), and Leiden, The Netherlands (pNL II) (Wyss, 1997). The *P. lagenophorae* line used in this study (rCH I) originated from a single-aeciospore culture from spores collected from pCH I. Before application, aeciospores were stored in Eppendorf vials at −18°C. The plant and the rust lines, the latter grown on the corresponding plant line, were maintained in a climate room with a 16 h photoperiod (130 μmol/m^2/s^1) at 23°C, and 8 h dark at 17°C with 60−80% relative humidity (RH).

Herbicides

Commercial formulations of glyphosate (*N*-(phosphonomethyl)glycine, Roundup, 31% a.i.; Monsanto, St. Louis, MO), linuron (3-(3,4-dichlorophenyl)-1-methoxy-1-methylurea, Afalon, 50% a.i.; Siegfried Agro, Zofingen, Switzerland), 2,4-D ((2,4-dichlorophenoxy)-acetic acid, 2,4-D-Liquid, 35% a.i.; Plüss-Stauffer, Oftringen, Switzerland), and MCPP ((*R*)-2-(4-chloro-*o*-tolyloxy)propionic acid, Mecoprop-P, 49.2% a.i.; Plüss-Stauffer), were used in the experiments.

Herbicide Effects on Spore Germination (in Vitro Study)

Herbicide stock solutions were prepared at recommended field rates and diluted with the appropriate amount of 1% water agar at 50°C resulting in the following final concentrations: 1X (field rate), 0.25X, 0.025X, and 0.0125X. Water volumes of 400, 500, and 1000 liter/ha were used for MCPP, glyphosate, 2,4-D, and linuron, respectively. This corresponded for glyphosate as 1800 μg a.i./ml (1X; 2.5 liter/ha) , 450 μg a.i./ml (0.025X), 45 μg a.i./ml (0.025X), and 22 μg a.i./ml (0.0125X); for linuron as 1000 μg a.i./ml (1X; 2 kg/ha), 250 μg a.i./ml (0.25X), 25 μg a.i./ml (0.025X), and 12.5 μg a.i./ml (0.0125X); for 2,4-D as 800 μg a.i./ml (1X; 2 liter/ha), 200 μg a.i./ml (0.25X), 20 μg

a.i./ml (0.025X), and 10 μg a.i./ml (0.0125X); and for MCPP as 2700 μg a.i./ml (1X; 2 liter/ha), 675 μg a.i./ml (0.25X), 67.5 μg a.i./ml (0.025X), and 33.8 μg a.i./ml (0.0125X). Water agar (1%) was used as a control.

Sterile glass slides were covered with the prepared water agar-herbicide mixture or water agar only. There were three replicated slides per herbicide concentration as well as for the control. The resulting 75 slides were transferred, each into a petri dish, and stored overnight at 4°C.

Ten milligrams of aeciospores was applied dry onto these slides by means of a settling tower (modified from Eyal *et al.* (1968)). Afterward the slides were placed on moist filter paper in a tray, covered with a plastic film, and incubated for 15 h at 18°C in the dark. Spore germination was stopped by coating the agar surface with lactophenol-cotton-blue. One hundred and fifty spores were counted and spores were considered germinated when the germ tube was longer than the diameter of the spore. In addition, any abnormal development of germ tubes was recorded. The experiment was conducted twice.

Herbicide Effects on the Infection Process (in Planta Study)

Peat pots (5 × 5 × 5 cm) filled with nutrient-amended peat (Floragard TKS2; Floragard, Oldenburg, Germany) were prepared and watered. Seeds were assigned randomly to the peat pots. One seed was placed in a pot and 120 pots were used for each plant line. The pots were placed in a climate chamber with growth conditions described above. Plants at the 4-leaf stage were selected for the experiment and 12 plants were used as replicates.

Plants were treated with sublethal rates of linuron (25 μg a.i./ml; 0.025X) and 2,4-D (20 μg a.i./ml; 0.025X) or tap water as a control in a spray chamber using spray tins (Birchmeier Aerospray 200, Künten, Switzerland). The *in vitro* study had shown that this rate is the highest concentration of these herbicides that allowed spore germination. A water volume of 400 liter/ha applied at 2.04 kg/cm^2 pressure with even spray nozzle delivery (Birchmeier Aerospray 200, Künten, Switzerland) was used. Half of the plants were sprayed 48 h before inoculation with the rust fungus and transferred back into the climate room with the conditions described above. The remaining plants were treated immediately (0 h) before inoculation with the rust fungus but after the plant surfaces had dried. This experiment was repeated once.

Aeciospores were applied dry onto the plants using the settling tower. In each experiment, four agar slides covered with 2% agar were placed between the pots to determine the spore density and germination after the incubation period. In the first experiment, spore density on agar slides was determined as 881 ± 74 spores/cm^2 (mean ± SE) and germination as 52% ± 2 (mean ± SE). In the second experiment, spore density was 743 ± 55 spores/cm^2 (mean ± SE) and germination 42% ± 4 (mean ± SE). Plants were then enclosed in a transparent plastic bag to retain humidity and placed in an incubation chamber for 15 h in the dark at 18°C. After the plastic bag was removed, plants were grown under controlled conditions as described above.

The first or second true leaf was cut off 48 h after inoculation and one leaf disc of 0.28 cm^2 was punched out as described in Wyss and Müller-Schärer (1999). There were 12 replicated plants per treatment. The leaf discs were stained to distinguish the infection structures of *P. lagenophorae* and examined with a fluorescence microscope following the method of Kuck *et al.* (1981). Infection stages, including spores deposited (N_1), germinated spores (N_2), appressoria (N_3), penetration pegs (N_4), intraepidermal vesicles (N_5), and primary hyphae (N_6) were counted on each leaf sample and the fractions of successive infection stages of rust development were computed as

$$\text{gsp/sp} = \frac{N_2 + N_3 + N_4 + N_5 + N_6}{N_1 + N_2 + N_3 + N_4 + N_5 + N_6} \quad [1]$$

$$\text{app/gsp} = \frac{N_3 + N_4 + N_5 + N_6}{N_2 + N_3 + N_4 + N_5 + N_6} \quad [2]$$

$$\text{peg/app} = \frac{N_4 + N_5 + N_6}{N_3 + N_4 + N_5 + N_6} \quad [3]$$

$$\text{ves/peg} = \frac{N_5 + N_6}{N_4 + N_5 + N_6} \quad [4]$$

$$\text{ph/ves} = \frac{N_6}{N_5 + N_6}, \quad [5]$$

where gsp is germinated spores, sp is total number of spores, app is appressoria, peg is penetration pegs, ves is intraepidermal vesicles, ph is primary hyphae, and N_1–N_6 are the numbers of the various infection stages as defined above. The stage referred to here as penetration peg (peg) is the formation of a glucan peg that connects the very small and hardly visible penetration peg to the neck of an intraepidermal vesicle (Wyss and Müller-Schärer, 1999). Ten days after inoculation when sori were open, the remaining leaf of the first leaf pair was cut off. Total leaf area and leaf area occupied by mycelium were measured using image analysis (Quantimet 500 Leica, Glattbrugg, Switzerland). Disease severity, expressed as the fraction of leaf area occupied by mycelium, was computed.

TABLE 1

Analysis of Variance for Germination of Aeciospores of *P. lagenophorae* Exposed to Selected Herbicides at Different Rates

Effect	*df*	*F*
Experiment	1	65.94***
Herbicide (*H*)[a]	3	31.80***
Herbicide rates (*R*)[b]	4	113.64***
H × *R*	12	10.91***
Residual	98	
Total	11	
	9	

[a] Herbicides used were linuron, glyphosate, 2,4-D, and MCPP.
[b] Five herbicide rates were used: recommended field rate (1X), 0.25X, 0.025X, 0.0125X, and water as a control.
*** $P < 0.001$.

Statistical Analysis

Data were analyzed using analysis of variance (ANOVA) with the General Linear Models procedure of the Statistical Analysis System (SAS, 1996). The repeated experiments showed similar effects of the treatment factors on germination and the infection parameters assessed and were treated as a blocking factor (in time) (Tables 1 and 2). However, the means, compared using Scheffé's *F* test, are given separately for the two experiments (Figs. 1, 2, and 3). Bartlett's test was performed to test the homogeneity of variance. Variances were balanced with $\log_{10}(x + 1)$ transformation.

RESULTS

Herbicide Effects on Spore Germination

The analysis of variance indicated highly significant effects of the main factors, herbicide and herbicide rate, and their interactions on germination of aeciospores (Table 1). The overall percentage of germination varied in the experiments and was higher in Experiment 2 (Fig. 1). Germination was suppressed completely at the recommended field rate (1X) as well as at 0.25X (Fig. 1), except for 2,4-D at 0.25X which still allowed germination in both experiments. At highly diluted herbicide rates of 0.0125X and 0.025X, glyphosate significantly reduced germination compared to the other chemicals at the same rates ($P < 0.001$ and $P < 0.01$, respectively, Scheffé's *F* test).

Abnormal growth features of germ tubes were seen with 2,4-D at 0.25X and 0.025X, with linuron at 0.025X, and with glyphosate at 0.0125X. Germ tubes clearly were stunted and shorter in comparison with germ tube development on the control slides (data not shown).

Herbicide Effects on the Infection Process

Disease severity and transition from appressorium to penetration peg (peg/app) varied between the two experiments; spore deposition did not (Table 2). Spore deposition was only affected by the factor herbicide. A significant effect of herbicide also was demonstrated for the transition from appressorium to penetration peg. Moreover, there was a significant effect of plant line and of the interaction of plant line by herbicide timing. Disease severity was influenced by plant line, but not by herbicide (Table 2).

In both experiments, leaves treated with 2,4-D at 0.025X had significantly fewer spores deposited than leaves treated with water ($P < 0.05$, Scheffé's *F* test) (Fig. 2A). A similar, but weaker effect was also observed for linuron in Experiment 2. Linuron, moreover, significantly reduced the formation of penetration pegs compared to 2,4-D and water ($P < 0.05$, Scheffé's *F* test, Fig. 2B).

Resistance at the stage of disease severity, defined as a difference between plant lines, was only found in Experiment 1 when data were plotted separately for both experiments (data not shown).

The proportion of germinated spores was lower in Experiment 2 compared to Experiment 1 (Fig. 3). The most sensitive transition of the infection process, peg

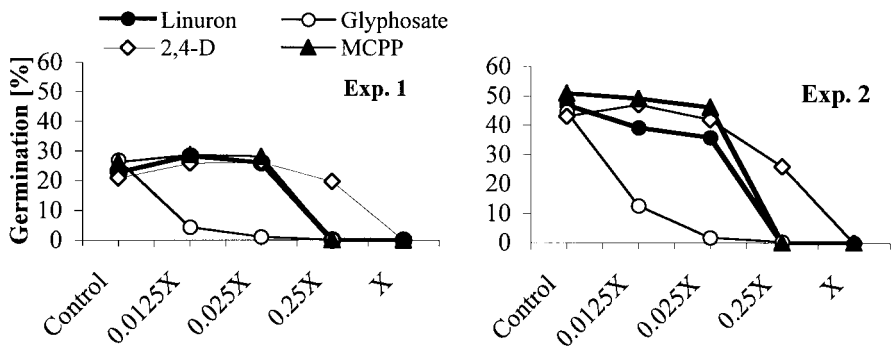

FIG. 1. Effect of herbicides on germination of aeciospores of *P. lagenophorae*. The herbicides linuron, 2,4-D, glyphosate, and MCPP were used at field (X) and sublethal rates (0.0125X, 0.025X, and 0.25X). A water control was included. The experiment was performed twice successively.

TABLE 2

Influence of Herbicide, Herbicide Timing, and Plant Line on Spore Deposition, Transition from Appressorium to Penetration Peg Formation (Peg/App), and Disease Severity of *P. lagenophorae* on *S. vulgaris*

	Spore deposition[a]		Peg/app[a]		Disease severity[b]	
Factor or interaction	*df*	*F*[c]	*df*	*F*	*df*	*F*
Experiment	1	0.01	1	4.24*		10.11**
Herbicide (*H*)	2	3.17*	2	3.34*	2	1.70
Herbicide timing (*T*)	1	1.29	1	2.15	1	0.62
Plant line (*P*)	1	0.04	1	6.13*	1	8.23**
$T \times P$[d]	1	0.82	1	5.91*	1	0.83
Residual	275		261		270	
Total	287		273		281	

[a] Spore deposition and the transition from appressorium to penetration peg were determined 48 h after spore application.
[b] Disease severity was determined 10 days after spore application.
[c] ANOVA was carried out on $\log_{10}(x + 1)$-transformed data.
[d] Only interactions that were significant are listed.
* $P < 0.05$.
** $P < 0.01$.

formation (peg/app), was affected by the use of linuron at 0.025X (Fig. 3). After peg formation, further development of infection structures was likely and showed the same pattern in the two experiments (Fig. 3).

DISCUSSION

This study was carried out to determine whether selected herbicides adversely affect the activity of *P. lagenophorae in vitro* and on its host, *S. vulgaris*, and if the susceptibility to the rust fungus might be increased by sublethal herbicide rates. The *in vitro* compatibility assay was used to quantify the effects of the herbicides on spore germination. Component analysis is used here for the first time to define the influence of

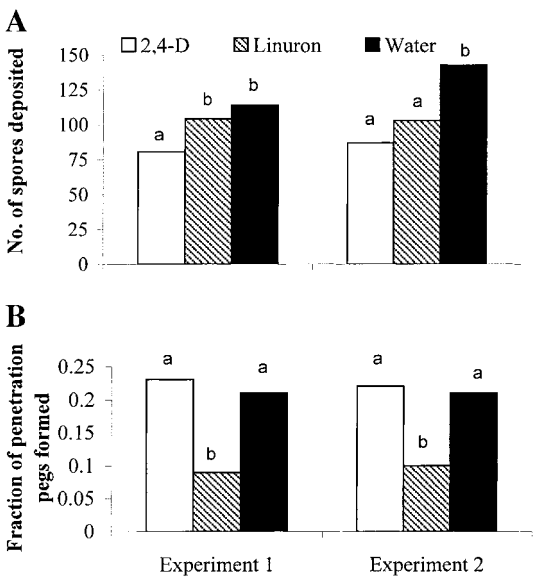

FIG. 2. Influence of two herbicides (applied at a sublethal rate of 0.025X, X being the field rate) and a water control on the infection of *S. vulgaris* by *P. lagenophorae*. (A) Spore deposition and (B) transition from appressorium to penetration peg (peg/app) formation. Herbicides and the water control were applied 48 or 0 h before application of the rust fungus. A leaf area of 0.28 cm^2 was assessed. Nontransformed data are shown. Different letters within experiments indicate significant differences at $P < 0.05$; Scheffé's *F* test. The experiment was performed twice successively.

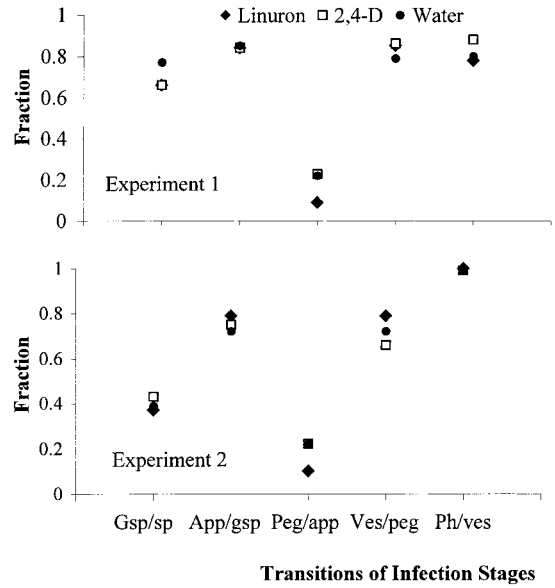

FIG. 3. Quantification of the infection process of *P. lagenophorae* on *S. vulgaris* treated with a sublethal rate (0.025X) of linuron and 2,4-D, and water control. Entries are mean fractions of two herbicide application timings (48 and 0 h before spore inoculation) and two plant lines (pCH I and pNL II), each with 12 replicated plants (total of 48 plants per herbicide or water control). Nontransformed data are shown and the experiment was performed twice successively. Infection structures were analyzed on a leaf area of 0.28 cm^2 48 h after inoculation. gsp, spores germinated; sp, total spores; app, appressoria; peg, penetration pegs; ves, intraepidermal vesicles, and ph, primary hyphae.

herbicides on successive phases within the infection process as suggested by Wyss and Müller-Schärer (1999). Component analysis enables determination of the stages of the infection process at which defense mechanisms of the plant become effective and reveal the stage of the infection process at which resistance mechanisms might be overcome (Wyss and Müller-Schärer, 1999; Zadoks and Schein, 1979).

The overall germination level differed between the two experiments. It is assumed that aeciospore maturity, constitutive dormancy, and the method of aeciospore storage may influence spore germination and quality (Littlefield, 1981). Such differences between the spores used in the two experiments may have influenced the differential germination.

All herbicides tested were highly toxic to spore germination at the recommended field rate (1X). At 0.25X they suppressed spore germination, with the exception of 2,4-D, which showed similar germination rates as the control (Table 1). Glyphosate inhibited germination even at 0.0125X. These results agree with those of Liu *et al.* (1991) who demonstrated poor germination of sporangia and radial growth of *Pythium ultimum* Trow at 10 and 100 μg/ml rates of glyphosate. Charudattan (1986) reported that there was a concentration-dependend growth inhibition of *Cercospora rodmanii* Conway, a biocontrol agent for *Eichhornia crassipes* (Mart.) Solms, by 2,4-D. There was a 50% reduction in colony growth by 2,4-D at 200% of the label rate, the highest concentration tested. Interestingly, sporulation of the same fungus was stimulated at concentrations of 100, 50, 25, and 13% of the label rate used. In field studies using *Phoma proboscis* Heiny to control *Convolvulus arvensis* L., 2,4-D and MCPP were compatible with the fungus at a sublethal rate of 18 g a.i./ha (Heiny, 1994). This rate is equal to the concentration we used for 2,4-D in the *in planta* study. In Grant *et al.* (1990) herbicides at concentrations from 0.01 to 2 times the recommended field rate that control broadleaf, grass weeds, and both types of weeds were tested on germination of spores of *Colletotrichum gloeosporioides* (Penz.) Sacc. f. sp. *malvae.* With linuron spore germination also declined as concentrations increased. The ester and the amine salt of 2,4-D, however, had no significant effects on spore germination in their experiments.

In our experiment, leaves of both lines of *S. vulgaris* rolled up 24 h after application with linuron and 2,4-D. This observation may explain the reduced deposition of aeciospores compared to the water control in both experiments. The leaves returned to normalcy at the time of disease severity measurements, i.e., 10 days after spore application.

Although linuron did not inhibit germination of aeciospores at 0.025X (Fig. 1), growth features of the rust fungus were affected. This influence of linuron was evident on spore deposition and early infection structures within the host plant, with the most significant effect being on the reduced formation of penetration pegs. This transition from appressorium to penetration peg was most sensitive as already pointed out for this pathosystem by Wyss and Müller-Schärer (1999). Nevertheless, the influence of linuron was apparently too weak to affect further fungal development.

Timing of herbicide application seemed to have no influence on the germination and infection steps. Application of linuron and 2,4-D 48 h before rust application delayed fungal development in a similar way as the application done at 0 h. Also, there was no interaction among plant line and herbicide that would indicate that resistance to the rust fungus was overcome (Table 2). Moreover, there was no resistance detected in Experiment 2 (data not shown).

CONCLUSIONS

The tested herbicides at recommended field rates adversely affected spore germination of *P. lagenophorae.* Thus, a joint application of the rust fungus with the herbicides is impractical. A sequential application might therefore be envisaged but further experiments are needed to prove its practicability. Herbicide application before or at spore inoculation revealed similar results and does not contribute to higher aggressiveness of the rust fungus. Sublethal rates of the herbicides did not help to overcome resistance at peg formation and thereby to increase host susceptibility and the biological control effect. An effective application of *P. lagenophorae* with these herbicides is possible only if the herbicides are used at sublethal rates of 0.025X or lower. However, these low rates might not have a controlling effect on *S. vulgaris* and other weedy plants.

The combined use of a spore germination test with a subsequent component analysis of the infection process (as illustrated in this study) to investigate interactions between chemical and microbial hebicides is a helpful tool to study integrated weed control systems.

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